Docket No. 47624-DIV (71417)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: J. Isner et al.

SERIAL NO.: 09/698,323 EXAMINER: Dr. Quang Nguyen

FILED: October 27, 2000 GROUP: 1636

FOR: COMPOSITIONS AND METHODS FOR MODULATING VASCULARIZATION

THE HONORABLE COMMISSIONER OF PATENTS AND TRADEMARKS
WASHINGTON, DC 20231

SIR:

DECLARATION PURSUANT TO 37 CFR 1.131

The undersigned declares as follows:

1. I, Takayuki Asahara, am a co-inventor of the above-identified application (hereafter the "subject application"). I am the Director of Regenerative Medicine and Research at the Kobe Institute of Biomedical Research and Innovation, RIKEN Center of Developmental Biology, Kobe, Japan. I am also a Professor in the Department of Physiology at Tokai University School of Medicine, Kanagawa, Japan.

2. As I understand it, the subject application discloses and claims, among other things, a method for inducing formation of new blood vessels in a mammal that has chronic or acute ischemia. The method involves, for example, administering to the mammal an effective amount of granulocyte-macrophage colony stimulating factor (GM-CSF) to help form the new blood vessels.

3. I reviewed an Office Action ("Office Action") from the United States Patent & Trademark Office dated May 20, 2003 issued in connection with the subject application. As I understand the Office Action, the patent Examiner rejected claims 50-52, 55-63, 65-68, 70, 72-79 and 81 as obvious over US Pat. No. 5,880,090 to

Hammond et al. ("Hammond"), in view of Asahara et al. (Science 275: 964 (1997); "Asahara") or US Pat No. 5,980,887 to J. Isner et al. ("Isner"). I understand that Hammond has an effective filing date of September 19, 1997.

4. I have read Hammond and am familiar with the Asahara and Isner references. As I understand the Hammond patent, it discloses a method of enhancing endothelialization of a synthetic graft by administering GM-CSF, for instance, to increase adherence of endothelial progenitor cells to the graft.

5. The invention described and claimed in my subject application was conceived and reduced to practice in the United States prior to September 19, 1997.

6. For example, and well before September 19, 1997, I and my co-inventor prepared a draft manuscript entitled "Regulation of Neovascularization with Ischemia- or Cytokine-induced Mobilization of Bone Marrow-derived Endothelial Progenitor Cells" (hereinafter "Manuscript"). Among other things, the Manuscript reported results of experiments in which we induced formation of new blood vessels in subject mammals by administering GM-CSF.

7. For example, and well before September 19, 1997, I and my co-inventor induced formation of new blood vessels by administering GM-CSF to subject mammals. Attached as **Exhibit 1** is a true and accurate copy of an Abstract taken from the Manuscript with dates deleted. The Abstract reports, among other things, that we pre-treated rabbits with GM-CSF, subjected them to ischemic surgery, and then observed extensive new blood vessel growth and better ischemic limb blood pressure.

8. In addition, and well before September 19, 1997, I and my co-inventor showed that it was possible to increase formation of new blood vessels by

administering GM-CSF to a mouse and then to monitor that new growth in a mouse. Attached as **Exhibit 2** is a true and accurate copy of a section taken from the Manuscript with dates and irrelevant information redacted. It discloses, among other things, that we pre-treated mice with GM-CSF, inserted a pellet with vascular endothelial growth factor (VEGF) into those mice and observed significant new blood vessel growth in a mouse cornea assay.

9. **Exhibit 2** also discloses, among other things, that well before September 19, 1997, I and my co-inventor understood that GM-CSF could be used to treat ischemic vascular disease. More specifically, we appreciated that GM-CSF could be used to treat chronic ischemic by inducing new blood vessel growth. **Exhibit 2** also reports details about how we pre-treated rabbits with GM-CSF and induced new blood vessel growth in that mammal as determined by a rabbit hindlimb ischemia model.

10. I and my co-inventor used a variety of assays to induce and monitor blood vessel formation after administration of GM-CSF well before September 17, 2002. Attached as **Exhibit 3** is a true and accurate copy of a section taken from the Manuscript with dates and irrelevant information redacted. It reports, among other things, details about how to make and use the rabbit ischemic hindlimb and mouse corneal neovascular assay models.

11. I and my co-inventor used these and other assays well before September 19, 1997 to monitor the effect of administering GM-CSF on new blood vessel growth. Attached as **Exhibit 4** is a true and accurate copy of a section taken the Manuscript, with dates and irrelevant information redacted. It discloses, among other things, how we used GM-CSF to induce new blood vessel growth in the murine cornea model. It also reports how we used GM-CSF to induce new blood vessel growth in mice ("mouse model) and rabbits ("rabbit model") subjected to ischemic conditions.

12. Well before September 19, 1997, I and my co-inventor learned that it was possible to induce new blood vessel growth in mammals by administering GM-CSF before and after inducing ischemia.

13. For example, **Exhibit 4** further shows, among other things, that we administered recombinant human GM-CSF to rabbits a week before ischemic surgery to induce new blood vessel growth in those mammals (GM-CSF group-1). **Exhibit 4** also shows that we administered the GM-CSF to rabbits that already had ischemia (chronic ischemia model).

14. **Exhibit 4** also discloses, among other things, how we monitored limb blood pressure, vasomotor activity, and obtained angiographic scores in the mouse and rabbit models to document enhanced new blood vessel growth following GM-CSF administration to these mammals.

15. I and my co-inventor developed methods to help monitor the extent of new blood vessel growth following administration of GM-CSF before and after induction of ischemia in test subjects. Attached as **Exhibit 5** is a true and accurate copy of a section taken from the Manuscript with dates and irrelevant information redacted. It shows, among other things, how we assessed new capillary density and capillary/muscle fiber ratios in response to administration of GM-CSF to the mammals.

16. I and my co-inventor analyzed our results from administering GM-CSF to subject mammals to induce new blood vessel growth before or after induction of ischemia. For instance, attached as **Exhibit 6** is a true and accurate copy of Figures 4A-C from the Manuscript with dates redacted. It shows, among other things, that administration of GM-CSF induced new blood vessel growth in the rabbit hindlimb ischemia model.

17. In particular, Figures 4A-B of **Exhibit 6** show that treatment of rabbits with GM-CSF enhanced EPC kinetics. These results were taken to be indicative of the new blood vessel growth in the rabbits according to our method. Figure 4C shows results of a physiological assessment of rabbits that received treatment with GM-CSF according to our method. The results indicate an increase in blood pressure ratio (ischemic to healthy limbs) which we took as consistent with induction of new blood vessels in response to the GM-CSF we gave to these animals.

18. I and my co-inventor Jeff Isner (now deceased) conceived of and practiced our method of inducing formation of new blood vessels by administering GM-CSF to subject mammals well in advance of September 19, 1997. In particular, and well in advance of that date, we induced formation of new blood vessels in mammals with chronic ischemia by administering GM-CSF to those mammals.

19. I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: _____

_____
Takayuki Asahara

#346097

ABSTRACT

Endothelial progenitor cells (EPCs) have been isolated from circulating CD34 antigen positive mononuclear cell in human peripheral blood and shown to be incorporated into foci of neovascularization, consistent with post-natal vasculogenesis. We investigated the hypothesis that EPC kinetics in circulation may have significant role in control of neovascularization, and hematopoietic mobilization with cytokines, such as granulocyte macrophage-colony stimulating factor (GM-CSF), known to enhance hematopoietic progenitors mobilization into peripheral blood, may enhance EPC kinetics and contribute to neovascularization in severe ischemia. The Sca-1 antigen-positeive population (Sca-1(+)) for mouse and the antigen-negative population of T-, B- lymphocyte and monocyte (TBM(-)), CD5-/Igμ-/CD11b-, for rabbit were proven as EPC-enriched fraction. Mouse and rabbit hindlimb ischemic model demonstrated increased frequency of EPC in circulation (day 7; 394% in mice, 536% in rabbit, compared to day 0), and enhanced EPC differentiation with assay culture of peripheral blood mononuclear cell (day 7; 204% in mice, % in rabbit, compared to day 0), throughout 3 to 14 days, with a peak at 7 days after ischemic surgery. The ischemic hindlimb model of mice, transplanted with LacZ, over-expressing bone marrow, and rabbits, with fluorescence labelled EPCs derived from TBM(-), demonstrated the significant role in neovascularization in severe ischemic lesion, showing its colonization, sprout and capillary formation. GMCSF pretreated rabbits, followed by ischemic surgery, demonstrated significant increase in circulating EPC population (419% at day0, 151% at day7) and enhanced EPC differentiation than control group from the day of (i.e. prior to) surgery (day 0), up to day 7. Morphometric analysis of capillary density presented extensive neovascularization induced by GM-CSF pretreatment than control group (249 vs 146/mm^2, $p<0.01$), as well as improved ischemic/normal limb blood pressure ratio (0.71 vs 0.49, $p<0.01$). These data suggest that EPC kinetics in circulation is enhanced in severe ischemic individual, and the fathermore extention of EPC mobilization, induced by GM-CSF, can augment neovascularization in ischemic lesions.

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GMCSF, which stimulates hematopoietic prognitor cell (*[Socinski M, *Lancet* 1988] [Gianni A, *Lancet* 1989]) and myeloid lineage cell (*[Clark S, *Science* 1987] [Sieff C, *J Clin Invest* 1987]), as well as non-hematopoitic cells including BM stroma cell (*[Dedhar S, *Proc Natl Acad Sci USA* 1988]) and EC (*[Bussolino F, *J Clin Invest* 1991]), was used for cytokine-induction of hematopoietic mobilization. To avoid direct mitogenic effect of GMCSF on EC, cytokine was administered for 7 days prior to stimulus for neovascularization. [De novo vascular formation was initially examined in mouse cornea assay. GMCSF-pretreated mice (rmGMCSF; 500ng/day i.p.) showed enhanced EPC kinetics (XX vs XX) at day 0 prior to VEGF pellet insertion and consequently more augmented neovascularization at day 6 than control mice (Fig. 4).] To realize if

cytokine-induced EPC mobilization could enhance neovascularization in severe ischemic tissue, the rabbit hindlimb ischemia model was employed. GMCSF pretreated rabbits (rhGMCSF; 50μg/day s.c.) presented initial increase in EPC-enriched cell population (189% compared to control animals) and enhanced ability of EPC differentiation (421% compared to control) at day 0, of (i.e. prior to) surgery, and gradual enhancement of EPC kinetics after ischemia with a peak at day 7 (240% in frequency and 151% in differentiation). Morphometric analysis of capillary density presented extensive neovascularization induced by GMCSF pretreatment than control group (249 vs 146/mm^2, $p<0.01$), as well as improved ischemic/normal limb blood pressure ratio (0.71 vs 0.49, $p<0.01$).

These resutls indicates GMCSF exerts potent stimuli on EPC kinetics and such a cytokine-induced EPC mobilization can enhance neovascularization in severe ischemia condition and de novo vascularization in avacular area. The concept of cytokine-induction of EPC mobilization could be a novel and promissing strathegy for clinical application to ischemic vascular disease, which lacks efficient blood supply. In the case, GMCSF will have not only effect on enhancement of EPC kinetics, but also direct mitogenic influence on EC. Actually, we have tried administration of GMCSF in chronic ischemic hindlimb model (10 days after ischemia) and recognized significant increase in physiological (blood pressure ratio, endothelium-dependent and -independent blood flow measurement) and histologic (capillary density) analyses 40 days after ischemic sugery (Fig. X or data not shown).

Rabbit Ischemic Hindlimb Model

We used a rabbit ischemic hindlimb model described previously [Takeshita, 1993 #500]. A total of 20 New Zealand White rabbits (3.8-4.2 kg) (Pine Acre Rabbitry, Norton, MA) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xylazine (2mg/kg). A longitudinal incision was then performed, extending inferiorly from the inguinal ligament to a point just proximal to the patella. The limb in which the incision was performed was determined randomly at the time of surgery by the operator. Through this incision, using surgical loupes, the femoral artery was dissected free along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric, were also dissected free. After dissecting the popliteal and saphenous arteries distally, the external iliac artery and all of the above arteries were ligated with 4.0 silk (Ethicon, Sommerville, NJ). Finally, the femoral artery was completely excised from its proximal origin as a branch of the external iliac artery, to the point distally where it bifurcates to form the saphenous and popliteal arteries. Following excision of the femoral artery, retrograde propagation of thrombus leads to occlusion of the external iliac artery. Blood flow to the ischemic limb consequently becomes dependent upon collateral vessels issuing from the internal iliac artery.

Mouse Corneal Neovascularization Assay Model

We used age-mached (8wk) C57BL/6J male mice (Jackson Lab, Bar Harbor, ME) or BM transplanted mice (BMT/LZ) described above, for mouse corneal neovascularization models. All animals were anesthetized by intraperitoneal pentobarbital injection (160

mg/kg) for subsequent surgecal procedures. Corneal pockets were created with a modified von Graefe cataract knife in the eyes of mice. Into each pocket, a 0.34*0.34 mm sucrose aluminum sulfate (Bukh Meditec, Denmark) pellet coated with hydron polymer typeNCC (IFN Science, New Brunswick, NJ) containing 180-200ng of vascular endothelial growth factor (VEGF) was implanted. The pellet were positioned 1.0mm from the corneal limbus and erythromicin ophthalmic ointment (XXXX, XX) was applied to each operated eye. The corneas of all mice were routinly examined by slit-lamp biomicroscopy on postoperative days 5 through 6 after pellet inimplantation. Vessel length and clock-hours of neovascularization were measured on the sixth postoperative day when all corneas were photographed. After the measurements, mice received 500μg of Bandeiraea Simplicifolia lectin-1 (BS-1) conjugated with FITC (Vector Lab, Burlingame, CA), EC specific marker, intravenously and then sacrificed 30 minutes latter. The eyes were enucleated and fixed in 1% paraformardehyde solution. After fixation, the corneas were placed on glass slides and observed with fluorescent microscope.

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Study Design for GMCSF Effect on Circulating EPC Kinetics and Ischemia

The experiments were scheduled to show GM-CSF effect on EPC kinetics consequently to see vasculogenic contribution to neovascularization

Mouse cornea model Following recombinant murine GMCSF (0.5 μg per a day) or control saline i.p. injection for a week, begining at day -7 through day -1, C57BL/6J mice (n=5 each) received corneal assay operation at day 0 and were measured with length and width angle of neavasculartur at day 6. In situ BS-1 lectin staining was performed before sacrifice.

Mouse model The GMCSF group consisted of 3 BMT/LZ mice to which recombinant murine GMCSF (0.5 μg per a day) was injected intraperitonealy for a week, beginning 7 days before surgery. The ischemic control group consisted of 3 BMT/LZ mice received a saline solution for a week before surgery. The ischemic and contralateral healthy muscles were devided into two samples for frozen blocks and for X-gal staining with 2% paraformaldehyde fixation. The frozen sections were prepared for alkarin phosphatase staining and counted histologically for capillary density measurement.

Rabbit model The first experiment (study-1) was employed to show GM-CSF effect on hematopoietic cells consequently to see vasculogenic contribution to neovascularization. Animals with hindlimb ischemia were divided into 2 groups. The GM-CSF treatment

animals consisted of XX rabbits to which ricombinant human GM-CSF (70μg per a day) was injected subculaneously for a week, beginning 7 days before surgery (GM-CSF group-1). The ischemic control group consisted of XX rabbits received a saline solution for a week before surgery (Control group-1).

The whole animals were investigated at the day immediately before inintial injection (day -7), the day of ischemic surgery (day 0), and 3, 7, 14 days post-operatively (day 3, 7, 14), at which time peripheral blood was isolated from central ear artery. At each time point, 5 ml blood was isolated for cell counting and culture assay. All animals from each group were studied with ischemic to helthy limb blood pressure ratio and capillary density of ischemic muscles at necropsy on post-operative day 14.

The second experiment (study-2) was employed to investigate GM-CSF effect on neovascularization and collateral formation in chronic ischemic model. The ischemic hindlimb animals received rhGM-CSF (70μg per day) or saline solution begining 10 days after surgery (day10) with subcutaneous injection every day for a week. (GMCSF group-2 and Control group-2).

The both groups were investigated at day 10 and 40 post-operatively at which time lower limb blood pressure, vasomotor reactivity, and angiographic score were evaluated (vide infra). All animals from each group were sacrificed on post-operative day 40, and studied at necropsy.

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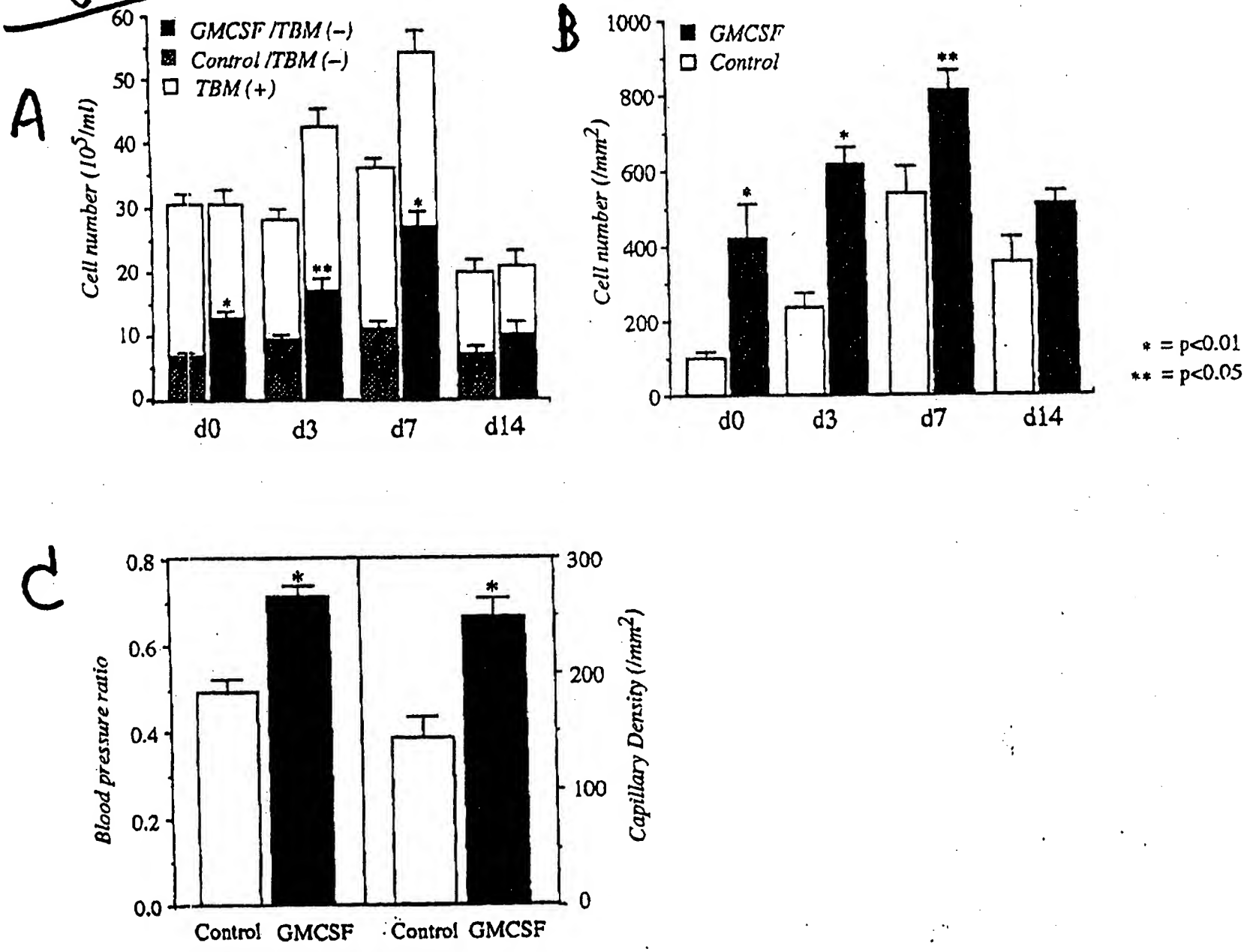
Capillary Density and Capillary/Muscle Fiber Ratio.

The extent of neovascularization was assessed by measuring the frequency of capillaries in light microscopic sections taken from the normal and ischemic hindlimbs. Tissue specimens were obtained as transverse sections from muscles of both limbs of each animal at the time of sacrifice. Muscle samples were embedded in O.C.T. compound (Miles, Elkhart, Ind.) and snap-frozen in liquid nitrogen. Multiple frozen sections 5 μm in thickness were then cut from each specimen so that the muscle fibers were oriented in a transverse fashion. The tissue sections were stained for alkaline phosphatase with an indoxyl-tetrazolium method to detect capillary endothelial cells as previously described [Baffour, 1992 #22; Flanagan, 1991 #156; Ziada, 1984 #572] and counterstained with eosin. Capillaries were counted under a 20X objective to determine the capillary density (mean number of capillaries/mm^2). Ten different fields were randomly selected for the capillary counts. To ensure that analysis of capillary density was not overestimated as a result of muscle atrophy or underestimated because of interstitial edema, the capillary/muscle fiber ratio was determined in each histologic section. The counting scheme used to compute the capillary/muscle fiber ratio was otherwise identical to that used to compute capillary density.

Statistical Analysis

All results are expressed as mean ± standard error (m±SE). Statistical significance was evaluated using unpaired Student's t test for comparisons between two means. A value of $p<0.05$ was interpreted to denote statistical significance.

Fig4.


A
GMCSF /TBM (−)
Control /TBM (−)
TBM (+)
Cell number (10^5/ml)
60
50
40
30
20
10
0
d0
d3
d7
d14
B
GMCSF
Control
Cell number (/mm^2)
1000
800
600
400
200
0
d0
d3
d7
d14
* = p<0.01
** = p<0.05
C
Blood pressure ratio
0.8
0.6
0.4
0.2
0.0
Capillary Density (/mm^2)
300
200
100
0
Control
GMCSF
Control
GMCSF